分类号_____ 密级_____
UDC_____ 编号_____

厦 门 大 学

博 士 后 研 究 工 作 报 告

Postdoctoral Research Report (Xiamen University)

Effects of B(a)P on immunomodulation, biochemical responses and DNA damage in the marine gastropod abalone *Haliotis diversicolor*

By
Dr. Singaram Gopalakrishnan

工作完成日期 **2007年11月——2010年4月**
报告提交日期 **2010年4月**

Molecular immunology and Toxicology Laboratory,
State Key Laboratory of Marine Environmental Science,
Xiamen University, Xiamen, Fujian 361005, China

厦门大学（厦门）

2010年4月

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作者签名：S. Gopalakrishnan 日期：2010 年 4 月 28 日

导师签名：王克坚　　日期：2010 年 4 月 28 日

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1.0 Abstract

It has been reported that organic xenobiotics commonly found as pollutants in the marine environment impair immune capabilities of marine organisms. The purpose of the present study was to investigate the effects of benzo(a)pyrene [B(a)P] on immunomodulation, oxidative stress and antioxidant defences in marine gastropods. In the first part of present study, the marine gastropod abalone *Haliotis diversicolor* was exposed to sublethal concentrations of B(a)P for shorter exposure periods and second part abalone exposed to environmentally realistic concentrations of B(a)P for longer exposure periods. During the study, alterations of hematological parameters like haemocyte count, haemocyte viability, protein content and immune components like phenoloxidase, phagocytosis and superoxide anion generation were measured. In addition, the changes in lysozyme activity, antibacterial activity due to the effect of B(a)P on abalone were analysed. B(a)P was found to decrease significantly the total number of circulating haemocytes. Intracellular superoxide anion generation and phenoloxidase significantly increased on exposure to B(a)P, whereas phagocytic activity was decreased significantly at higher concentration. Significant alterations were found in the uptake of neutral red and results of the present study showed that alteration in the immune function in abalone may be due to the generation of reactive oxygen species produced by the animal due to effect of B(a)P. The results are discussed in the light of elucidating the possible relationship between environmental contaminants and the immunological parameters studied.

During the study, sublethal effect of B(a)P on oxidative stress and modulation of antioxidant defenses were evaluated in different tissues of a *H. diversicolor*. Levels of

lipid peroxides (LOOH), thiobarbituric-acid reactive substances (TBARS), protein carbonyls (CP) and low- (L-SH) and high-molecular weight thiols (H-SH) were measured for oxidative stress. CP, LOOH and TBARS contents increased significantly in gill, kidney and hepatopancreas after B(a)P exposure. L-SH concentrations increased up to 2 fold in gill, kidney and muscle. The modulation of a suite of antioxidant defence enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione S transferase (GST), glutathione peroxidase (GPx), glutathione (GSH) in different tissues of abalone exposed to nominal concentrations of B(a)P for 7 d were observed. The activities of SOD and CAT were altered under B(a)P exposure. In most tissues, the activities of glutathione-utilizing enzymes (GPx, GST, GSH) were affected under B(a)P exposure. The GSH level decreased nearly 67 and 74 % in gonad and hepatopancreas whereas the same decreased approximately 40% in gill, kidney and mantle. B(a)P at 0.08 mg l^{-1} increased the level of GST in gill and kidney by 85% and 125%, respectively, whereas the same increased to 62% in hepatopancreas. Correlation analysis showing a relationship between oxidative stress and antioxidant defenses in abalone exposed to B(a)P was significant. However, during the long term exposure to environmentally realistic concentration of B(a)P, the antioxidant parameter showed no significant changes except in some tissues after 14 days exposure. Conversely, DNA damage increased in all the tissues after 28 d exposure. Overall the results indicate that the gill, kidney and hepatopancreas were the most sensitive organs to oxidative damage and thus suggest that *H. diversicolor* could be used as a suitable bioindicator of exposure to organic pollutant measuring activities of antioxidant enzymes.

In addition, the objective of this study was to investigate the sublethal effects of B(a)P on marker enzymes (EROD; LPO; AChE, phosphatases and transaminases in digestive gland, gill, gonad and hemolymph in the edible gastropod abalone *H. diversicolor*. Significant differences between control and B(a)P exposed abalone were observed for biomarkers enzymes studied. The four marker enzyme (ACP, ALP, ALT and AST) exhibited a similar pattern in all the tissue studied, while EROD and LPO showed induction in all tissues and AChE showed decreasing trends. In contrast, the hemolymph showed increasing in activities of marker enzymes except for AChE. The activity of ACP and ALP decreased nearly 33 and 24 % in digestive gland similarly ALT and AST activity decreased up to 25 and 69 % in digestive gland when the abalone were exposed to B(a)P at 0.08 mg l^{-1}. All the results were tested statistically and interpreted accordingly and the results indicate that the measurement of biomarker responses is technically feasible. The performance of each biomarker is assessed in the context of the role and advantages of selecting a battery of biomarkers for detecting contamination problems.

Keywords: Abalone, B(a)P, Immunotoxicology, Biomarkers and *H. diversicolor*

2.0 Introduction

Environmental pollution is a growing concern and, more importantly, pollution of the aquatic ecosystem is alarming. Marine pollution may be one of the reasons for disease incidence in marine organisms, due to adverse effects of pollutants on the physiology of the animal especially the immune system. Natural and man-made organic foreign compounds (xenobiotics) enter and are dispersed in aquatic ecosystems by various routes, including direct discharge, direct use, land run-off, atmospheric deposition, *in situ* production, abiotic and biotic movement, and food chain transfer (Livingstone et al., 1992). Many studies have clearly shown a possible relationship between various pollutants and the stress that it causes in animals, as well as diseases (Sinderman, 1993). The effects of a pollutant on animals may be a direct action or an indirect one. Most importantly, alterations in the homeostatic mechanisms, including the immune system are critical, since these predispose an animal to infection and subsequent disease (Fournier et al., 2000).

Environmental contaminant effects may result from direct toxic action on the tissues or from more subtle alterations in homeostatic mechanisms. The study of a range of components which comprise an integrated biological system like the immune system may therefore provide a sensitive and comprehensive measure of the health status of an organism, reflecting the degree of pollutant induced stress and thus give an early indication of disease susceptibility and, ultimately, survival (Pipe et al., 1999). B(a)P is a member of a class of compounds known as the polycyclic aromatic hydrocarbons (PAHs), in which the molecular structure includes two or more fused aromatic rings and adjacent rings share two or more carbon atoms. B(a)P is considered here because it is the

one of the important PAHs for which there are sufficient toxicological evidence to allow the setting of a guideline.

Many chemicals introduced into the environment as a result of industrial or agricultural activity have been implicated in ecotoxicological effects mediated via immunotoxic mechanisms in exposed populations. The dependence of immune responses on receptor binding renders the system vulnerable to interference by toxicants which may trigger inappropriate responses, whilst any toxicant which interferes with cellular energy metabolism and the production of ATP will subsequently reduce immune cell viability and effector functions (Buttgereit et al., 2000). Examination of the available information reveals that some species like bivalve molluscs have received a great deal of attention on immunotoxicity due to xenobiotics (Coles et al., 1994; 1995; Pipe et al., 1999 and Thiagarajan et al., 2006), while gastropod abalone have only been investigated in relation to ecological factor (Cheng et al., 2004a, b,c, d, e) . This is clearly a deficiency since a distorted view of the effects of immunotoxic chemicals in biota may have arisen (Galloway and Depledge, 2001).

Molluscs have been extensively used in marine pollution monitoring programmes and the gastropods that have a wide geographical distribution in coastal waters are known to readily accumulate pollutant and show various physiological and biochemical responses providing information on the general status of contamination in the coastal environment and health of the animal itself. A variety of bio-markers has been used to monitor the level of environment pollution (Nicholson, 2003; Siu et al., 2004; Nicholson and Lam 2005; Thiagarajan et al., 2006). Most studies on immunomodulation in

molluscs have shown drastic changes in animal's immune competence upon exposure to different categories of pollutants (Pipe et al., 1999; Pavlica et al., 2000; Sauve et al., 2002; Gagnaire et al., 2004). The immune defence system of molluscs is comprised of cell-mediated and humoral mechanisms, in which the haemocytes play a key role (Cheng, 1981). Antigenic challenge stimulates migration of haemocytes, followed by phagocytosis and intracellular degradation of the pathogen by means of lytic enzymes (Pipe, 1990) or the production of highly reactive oxygen metabolites (Pipe, 1992). Abalones are large algivorous marine gastropods, and the most commercially important species of gastropods in aquaculture. However, current studies on abalone immune functions are limited, and most of them mainly focus on the relationship between ecological factors or disease and the immune response of abalone (Malham et al., 2003, Cheng et al., 2004 a,b,c and Hooper et al., 2007). Until now to our knowledge there are no studies on the effects of organic pollutants concerning immune competence of the abalone. Thus, this investigation is the first to deal with immunomodulation in *H. diversicolor* exposed to sublethal concentrations of organic pollutant.

During metabolism of PAHs, reactive oxygen species (ROS) could be generated (Garcia-Martinez and Livingstone, 1995). Some of the major consequences of ROS production in biological systems are protein oxidation, enzyme inactivation and DNA damage (Kehrer, 1993).The steady-state level of ROS is the balance between production and decomposition, and an imbalance in these processes in favor of the former has been termed "oxidative stress" (Halliwell and Gutteridge,1999; Hermes-Lima, 2004). Enhanced production of ROS is thought to be an important mechanism of pollutant mediated toxicity in aquatic organisms (Livingstone, 2001). Oxidative stress can disturb

and damage many cellular processes, sometimes leading to cell death. To deal with the potential dangers of ROS, both molecular (i.e.GSH) and enzymatic (various antioxidant enzymes) antioxidant defenses have arisen. The enzymes SOD, CAT, GPx and GST are recognized as a key interacting line of defence against ROS and their products of attack (Halliwell and Gutteridge, 1999). Antioxidant enzymes thus play a crucial role in maintaining cell homeostasis and these antioxidant enzymes have been proposed as biomarkers of contaminant-mediated oxidative stress in a variety of marine organisms, and their induction reflects a specific response to pollutants (Cossu et al. 1997).

The PAH emanating either from natural sources or from anthropogenic activities interact with aquatic organisms. Many researchers have performed risk assessments and toxicological studies of PAHs (Akcha et al., 2000; Cheung et al., 2001; Perez-Cadahia et al., 2004; Pan et al., 2006; Sun et al., 2008). However, the mechanisms of PAH toxic effect on aquatic organisms are not clearly elucidated. It is known that oxidative stress is an important mechanism of toxicity induced by PAHs (Livingstone, 2001; Shi et al., 2005; Sun et al., 2006; Yin et al., 2007) and some studies have been reported that PAHs alter the function of antioxidant function in mussel (Livingstone et al., 1988, Livingstone,1998; Cheng et al 2001; Cheng et al 2004). However, studies related to oxidative stress and antioxidant defences have not been discussed in gastropod and similarly the toxicity effect of B(a)P and its influence on modulation of antioxidant enzymes in marine gastropod were left out until now.

It is well known that biochemical constituents and marker enzymes have been explored as potential biomarkers for different organisms in recent years. Their advantages are that biochemical and enzyme activities tend to be more sensitive, highly conserved

between species, less variable and often easier to measure as stress indices. In addition, these are the first detectable and moreover easily quantifiable responses to environmental changes and can serve as markers for both exposure and effect in organisms. Acetylcholinesterase (AChE) activity is considered as a biomarker in evaluating the effects of exposure to neurotoxic compounds in aquatic animals (Cajaraville et al., 2000). Recent studies revealed that environmental contaminants including metals and surfactants may inhibit cholinesterase activity in aquatic animals (Payne et al., 1996; Guilhermino et al., 1998). AChE activity has been used as a biomarker of neurotoxicity (Peakall, 1992). Though many studies focused on oxidative stress and antioxidant defense system in marine molluscs, a very few of these are directed to use the marker enzyme. Hence, similar studies become very important taking into consideration of additive of xenobiotic reaching the marine environment, arising through cluster of industries through the industrial areas.

Phosphatases and transaminases are the marker enzymes were used as a biomarker to study the impact of xenobiotic compounds (Vijayavel and Balasubramaniam, 2006). Phosphatase enzymes catalyse the hydrolysis of various phosphate-containing compounds and act as transphosphorylases at both acid and alkaline pH (Blasco et al., 1993). Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be modulated by the presence of pollutants (Cajaraville et al., 2000, Vijayavel and Balasubramaniam, 2006), whilst alkaline phosphatases are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells (Blasco et al., 1993). Aspartate transaminase (AST) activities used as indicators of stress and alanine aminotransferase (ALT) which have been

employed for diagnosing hepatic damage in vertebrates and invertebrates (Neff, 1985; Blasco and Puppo, 1999).

DNA structure and integrity may be altered due to chemical agents such as PAHs and some PAH have been categorized as promutagens (Johnson, 1992; Maria et al., 2002b).During the metabolism of PAHs by the cytochrome P450 (CYP)-dependent monooxygenase system may leads to the formation of oxyradicals (Livingstone et al 1993: DiGiulio et al.,1993). DNA strand breaks are potentially mutagenic lesions which have been proposed as genotoxic biomarkers for marine environment (Mitchelmore and Chipman, 1998). It has been well documented that B(a)P induced DNA damage in molluscs, however there is still a lack of comprehensive research on both time and dose dependent effects of exposure on marine gastropods hence in the present study DNA integrity has been chosen to study the effect of B(a)P on gastropod abalone.

It is well known that the cytochrome P450 (CYP) system consists of a family of enzymes which involve in the metabolism of xenobiotics and endogenous compounds and activates, inactivates and facilitates the excretion of lipophilic chemicals (Navas and Segner, 2000). Hahn, (1998) reported that the induction of cytochrome P4501A (CYP1A) is mostly associated with exposures to PAHs and halogenated aromatic hydrocarbons (HAHs). EROD induction is a sensitive indicator of environmental alterations due to xenobiotics and easily detectable, quantifiable responses to exposure (Stegeman 1992) also it represents the cumulative impact of xenobiotics, whether or not they are detected analytically. In recent years more studies were carried out to investigate changes on EROD activity in Zebra mussel (*Dreissena polymorpha*) exposed to different pollutants at laboratory conditions (Binelli et al. 2006). Many field and laboratory studies have

reported that EROD is a sensitive biomarker in detecting the effects of pollutants in aquatic environment (Lemaire-Gony et al., 1995; Viarengo et al., 1997; Wassenberg et al., 2002). However, only a few studies relating to EROD have been done in invertebrates especially in gastropods so more studies should be conducted to address this deficiency.

It has been reported that lipid peroxidation (LPO) is a good biomarker for cytotoxicity, reflecting the inability of antioxidant defenses, (Sole et al., 1995; Telli Karakoc et al., 1997) and earlier studies on mollusks reported LPO as a biomarker to assess the impact of xenobiotics. Due to their wide geographic distribution molluscs have been found to be useful as indicators and integrators of certain contaminants, in coastal and estuarine communities. The importance of molluscs by serving economic food source for humans, and ease in collection and maintenance under laboratory conditions and earlier reports on molluscs indicate that it can tolerate persistent toxic chemicals, such as PAH and PCB in greater extent than other organisms (Smolders *et al*., 2003; Cheung et al., 2001; 2004). Analysis of biomarker mentioned above and other stress responses in tissues of organisms exposed to PAH can help to understand the associated toxic mechanisms and to predict the degree of biological damage at higher levels of biological organization.

2.1 Objective of the study

The aim of the present study thus was to investigate the effects of B(a)P on the immune function of the gastropod *H. diversicolor*. The parameters studied were related to the ability of the blood cells to destroy invading pathogens and included changes in the number and character of the circulating haemocytes, phenoloxidase enzyme activity,

superoxide anion production, phagocytosis, uptake of neutral red, lysozyme activity and antibacterial activity.

In addition, this study also used a large set of biomarkers to identify specific and distinctive patterns of responses of *H. diversicolor* to B(a)P to further characterize the changes occurring in several indicators of oxidative stress during B(a)P exposure in abalone *H. diversicolor*. The accumulation of damage products of oxidative stress in different tissues of abalone was analyzed by measuring CP as products of the oxidative modification of proteins and TBARS as indicators of the oxidative modification of lipids. The latter method has been widely used to assess oxidative stress in many organisms from bacteria to mammals, whereas the former is not yet in common use (Bagnyukova et al. 2003). Activities of selected antioxidant and associated enzymes were assessed in parallel. These included CAT, SOD, GSH, GPx and GST. In addition, levels of both L-SH and H-SH groups which represent the sum of various soluble thiol-containing metabolites and thiol groups in acid-precipitable proteins were assessed. The present study also studied the interaction of marker enzymes in abalone in the digestive gland and other tissues of edible gastropod *H. diversicolor* with reference to enzyme activities of AChE, ACP, ALP, ALT, AST, DNA integrity, EROD and LPO levels. A multi-assay approach was adopted with a view to monitoring the overall health status of the organisms under contaminant stress.

3.0 Materials and methods

Animals

Live healthy *H. diversicolor* (55 ± 5 mm in shell length) obtained from the Zhangpu abalone farm of Fujian Province were acclimatized to the laboratory conditions with temperature 24± 1°C, salinity of 30±1‰ and pH 7.8 ±0.1 for seven days before experimentation. Animals were reared in 80 L PVC tanks containing 40 L seawater treated with sand filtration, kept on a natural daylight cycle and fed with the marine alga *Gracilaria tenuistipitata* during the acclimation and experimental period.

Chemicals

Benzo(a)Pyrene (purity, 99%), 3,4-Dihydroxy-L-phenylalanine (L-DOPA), *Laminarin digitata, Micrococcus lysodeikticus,* Nitro blue tetrazolium (NBT) and LPS from *E. coli,* , Thiobarbituric acid, butylated hydroxytoluene, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), 2,4-dinitrophenylhydrazine, cumene hydroperoxide, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), Acetylthiocholine Iodide, Malondialdehyde, p-nitrophenol, p-nitrophenyl phosphate, 2,4-dinitrophenylhydrazine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 7-Ethylresorufin, Resorufin and NADPH were obtained from Sigma (Sigma Chemicals, St. Louis, MO USA) and all other chemicals used were of analytical grade.

3.1 Benzo(a)Pyrene bioassay test

Acute toxicity (96h) study was carried out to determine the lethal (LC_{100}), median lethal (LC_{50}) and sublethal (LC_0) level of B(a)P to *H. diversicolor* by static renewal method (EPA/ROC, 1998). Stock solution of B(a)P was prepared at 1 part per thousand